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Review

In vivo growth of porcine reproductive and respiratory syndrome virus engineered nsp2 deletion mutants

Kay S. Faaberg[a,*], Marcus E. Kehrli Jr.[a], Kelly M. Lager[a], Baoqing Guo[b], Jun Han[c]

[a] Virus and Prion Diseases Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA 50010, USA
[b] Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA 50011, USA
[c] Department of Veterinary and Biomedical Sciences, University of Minnesota, Saint Paul, MN 55108, USA

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ABSTRACT

Prior studies on PRRSV strain VR-2332 non-structural protein 2 (nsp2) had shown that as much as 403 amino acids could be removed from the hypervariable region without losing virus viability in vitro. We utilized selected nsp2 deletion mutants to examine in vivo growth. Young swine (4 pigs/group; 5 control swine) were inoculated intramuscularly with one of 4 nsp2 deletion mutants (rΔ727–813, rΔ543–726, rΔ324–523, rΔ324–726) or full-length recombinant virus (rVR-2332). Serum samples were collected on various days post-inoculation and analyzed by HerdChek* ELISA, PRRSV real time RT-PCR, gamma interferon (IFN-γ) ELISA, and nucleotide sequence analysis of the entire nsp2 coding region. Tracheobronchial lymph node weight compared to body weight was recorded for each animal and used as a clinical measurement of viral pathogenesis. Results showed that all deletion mutants grew less robustly than full-length recombinant virus, yet all but the large deletion virus (rΔ324–726) recovered to parental viral RNA levels by study end. Swine receiving the rΔ727–813 mutants had a significant decrease in lymph node enlargement compared to rVR-2332. While swine infection with rVR-2332 caused a rapid rise in serum IFN-γ levels, the IFN-γ protein produced by infection with 3 of the 4 deletion mutant viruses was significantly reduced, perhaps due to differences in viral growth kinetics. The rΔ543–726 nsp2 mutant virus, although growth impaired, mimicked rVR-2332 in inducing a host serum IFN-γ response but exhibited a 2-week delay. Targeted sequencing showed that all deletions were stable in the region coding for nsp2 after one swine passage. The data suggested that the selected nsp2 deletion mutants were growth attenuated in swine, altered the induction of serum IFN-γ, an innate cytokine of unknown function in PRRSV clearance, and pointed to a domain that may influence tracheobronchial lymph node size.

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* Corresponding author at: USDA-ARS B-14, Virus and Prion Diseases of Livestock, National Animal Disease Center, 2300 Dayton Avenue, Ames, IA 50010, USA. Tel.: +1 515 663 7259; fax: +1 515 663 7458.
E-mail address: kay.faaberg@ars.usda.gov (K.S. Faaberg).

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1. Introduction

One of the most economically important diseases of swine worldwide is caused by porcine reproductive and respiratory syndrome virus (PRRSV) (Neumann et al., 2005). This virus, belonging to the *Nidovirales* order, *Arteriviridae* family of positive-sense single-stranded RNA viruses, can cause reproductive failure in pregnant sows, respiratory disease in growing swine and asymptomatic infection in boars. PRRSV is also subject to high frequency viral recombination, which has led to rapid evolution and the existence of a multitude of strains belonging to one of two genotypes, Type 1 (European-like) and Type 2 (North American-like). Growing evidence suggests the swine immune response is often ineffective against heterologous PRRSV strains primarily because of the nature of the virus itself (Faaberg et al., 2006; Lopez and Osorio, 2004; Plagemann, 2004a,b).

With the advent of reverse genetics for several strains of PRRSV, portions of the genome can be directly assessed for disease function. One such area is the region of the ORF1 replicase protein known as non-structural protein 2 (nsp2) (Snijder et al., 1995b; Tian et al., 2007; van Hemert and Snijder, 2008), the most variable section of the genome (Han et al., 2006). Based on considerable research on equine arteritis virus (EAV), the nsp2 protein is processed from the ORF1 protein by the action of the papain-like protease in nsp1β and the self-encoded PL2 cysteine protease, and acts as a co-factor for the nsp4 serine protease (3CL) in processing downstream viral cleavages (den Boon et al., 1995; Snijder et al., 1994, 1995a,b; van Hemert and Snijder, 2008; Wassenaar et al., 1997). In addition, EAV nsp2 induces double membrane vesicles, probably through its predicted transmembrane spanning regions, and serves as an anchor for the virus replication complex (Pedersen et al., 1999; Snijder et al., 2001). Nsp2 of both genotypes of PRRSV is considerably larger than that of EAV and contains, in addition to the signature motifs described above, an extended N-terminal hypervariable region (HV1) and a middle hypervariable region (HV2). HV2 can differ in length by up to almost 150 amino acids. The two hypervariable regions of recombinant Type 2 strain VR-2332 (rVR-2332) were shown to contain amino acid segments not essential for replication in cell culture (Han et al., 2007) and recently, the cysteine protease domain (PL2) of strain VR-2332 was shown to possess both *cis* and *trans* cleavage activities and to cleave an expressed nsp2–3 construct at or near a downstream the predicted conserved G|G|G tripeptide (nsp2 amino acid residues 1196–1198) (Han et al., 2009; Ziebuhr et al., 2000).

The PL2 protease may also interact directly with cellular proteins. Shown through bioinformatics to be related to the mammalian ovarian tumor domain (OTU) containing protease family, PRRSV PL2 enzyme is thought to be a new member of this class of novel cysteine proteases. The sequence conservation among over 100 OTU members centers on the motifs containing the putative catalytic Cys and His residues, which arterivirus PL2 proteases possess (Makarova et al., 2000). PRRSV nsp2, when expressed individually in transfected 293T cells, decreases the level of ubiquitin and interferon-stimulated gene 15 (ISG15) conjugates (Frias-Staheli et al., 2007), indicating that the PL2 protease may deubiquitinate ubiquitinylated proteins. Protein ubiquitination has been shown to play a critical role in innate and adaptive cellular immune system induction by regulating several aspects of antiviral immunity such as NF-kB signaling, toll-like receptor/interleukin I signaling, MHC class I and II antigen presentation, and induction of type I IFN by the cellular viral sensor retinoic acid-induced gene I (RIGI) (Liu et al., 2005). The OTU domain of EAV decreases the activation of an NF-kB-responsive promoter after TNFα treatment, which may indicate that the NF-κB signaling pathway could be compromised by the presence of the arterivirus PL2 protease (Frias-Staheli et al., 2007; Hayden et al., 2006; Lenschow et al., 2007).

Nsp2 has also been shown to possess B-cell epitopes in both genotypes of viruses (de Lima et al., 2006; Oleksiewicz et al., 2001) and to induce B-cell responses (Chen et al., 2010; de Lima et al., 2006; Fang et al., 2004; Johnson et al., 2007; Mulupuri et al., 2008; Yan et al., 2007). Recently, using a type I PRRSV infectious clone, six identified immunodominant nsp2 B-cell epitopes [ES2–ES7; Oleksiewicz et al., 2001] were deleted. Three of these small domain deletions (ΔES3, ΔES4 and ΔES7) allowed the recovery of viable virus. The deletion mutants showed different levels of peak viremia in swine, with ΔES3 showing increased viral load over the other two mutants as well as parental rescued virus. However, the B-cell responses were similar when measured by IDEXX HerdChek, and neutralizing antibody responses were varied among all swine. Thus, removal of these B-cell epitopes did not appear to have an effect on the antibody responses. Only IL-1β and TNFα expression levels were different, showing a much lower level of expression after cells were stimulated with the ΔES3 mutant than with the ΔES4 and ΔES7 deletion viruses, or with the parent virus. Importantly, no lung lesion data was given (Chen et al., 2010).

In this report, we used four nsp2 deletion mutant viruses derived from the infectious clone of Type 2 PRRSV strain VR-2332. Three of these deletion viruses (rΔ727–813, rΔ543–726, rΔ324–523) were shown to be roughly similar in their in vitro growth kinetics, in terms of plaque forming units/ml, compared to parent virus (rVR2332). The virus with the largest nsp2 deletion (rΔ324–726) grew more slowly and produced an atypical cytopathic effect. Each mutant produced a smaller plaque size than rVR2332, and the large deletion mutant (rΔ324–726) produced no discernable plaques (Han et al., 2007).

Strain VR-2332, when administered to healthy pigs under defined hygienic experimental conditions, often produces an extremely mild clinical disease, as was noted in this study. Here, infection of growing swine with rVR-2332 and four engineered nsp2 deletion viruses were used to assess their impact on viral replication and to examine potential changes in the host response. Serum samples taken at various times were examined for anti-PRRSV antibody, the level of virus or viral RNA detected, and the stability of the deletions. We found that anti-PRRSV antibody levels were quickly induced by all but the mutant virus with the largest nsp2 deletion (rΔ324–726), that all inoculated viruses would replicate animals but at different rates, and that the engineered nsp2 deletions were stably maintained. For examination of the innate immune response, we chose to survey serum IFN-γ, as a previous

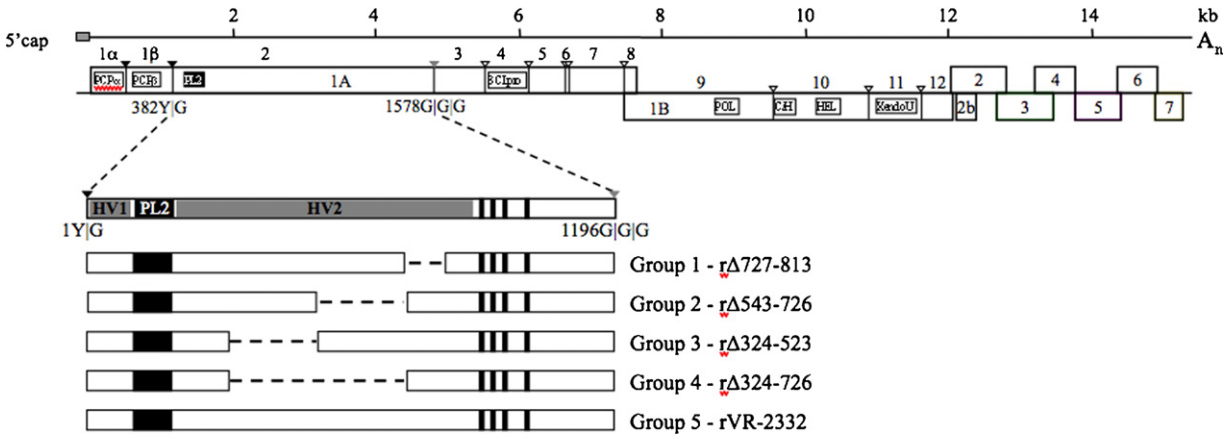

Fig. 1. Genome and PRRSV deletion mutant schematic. Illustration of treatment groups 1–5 used for deletion mutant growth analyses and pathogenesis assessment. Downward arrows indicate predicted cleavage sites processed by PRRSV encoded PCPα and PCPβ (papain-like cysteine protease α and β; ▼) chymotrypsin-like cysteine protease (PL2; ▼) and serine/3C-like protease (3CLpro; ▽). Other signature motifs are also indicated: POL, RNA dependent RNA polymerase; C/H, cysteine/histidine rich; HEL, helicase; XendoU, *Xenopus laevis* homolog poly(U)-specific endoribonuclease. The short black bars (⌘) indicate predicted nsp2 transmembrane regions. Hypervariable regions of nsp2 (HV1 and HV2), defined previously, are shown in solid grey (Han et al., 2006).

study had shown the level of this cytokine rose soon after infection with Type 2 PRRSV strains SDSU73 and JA142, peaked after 10 days and remained in the serum for approximately 3 weeks (Wesley et al., 2006). We found that similar high serum IFN-γ levels were induced by recombinant PRRSV strain VR-2332, but that the nsp2 deletion mutants differed from the parent virus in that all but one mutant did not elicit IFN-γ, and the mutant that did elicit this innate cytokine (rΔ543–726) showed a marked delay in doing so. Lastly, clinical severity was completed through lymph node weight to body weight measurements, as the ratio had been shown to be a reliable indicator of lymph node enlargement (Mengeling et al., 2003). In this study, only the smallest deletion mutant virus (rΔ727–813) resulted in decreased lymph node hyperplasia.

Previous reports of lymphoid hyperplasia and preferential proliferation of undiversified B cells during PRRSV infection have suggested a unique interaction between the virus and the host immune system (Butler et al., 2007; Lemke et al., 2004), and the identification of B-cell and T-cell epitopes in the nsp2 region bear this out (Chen et al., 2010; Fang et al., 2004). In the present study, results suggested that the change in viral growth kinetics of the engineered nsp2 mutant viruses of strain VR-2332 may be related to the quality of the host response, as was indicated previously with Type 2 strain JA142 (Greiner et al., 2000). Only the smallest nsp2 deletion resulted in a virus (rΔ727–813) that significantly reduced both the IFN-γ levels in serum and the degree of lymphadenopathy observed at necropsy, which corresponds to a deletion of only 4 predicted B-cell epitopes. Removal of B-cell epitopes located elsewhere in nsp2 reduced IFN-γ levels, but did not alleviate lymph node enlargement. Thus, no direct correlation between the numbers of nsp2 predicted B-cell epitopes and the level of lymph node involvement. However, removal of individual sections of nsp2 hypervariable region 2 was shown to have a differential effect on the viral kinetics and clinical disease phenotype.

2. Materials and methods

2.1. Cells and viruses

MARC-145 cells were cultured in minimum essential medium (EMEM, SAFC 56416C) with 10% fetal bovine serum at 37 °C, 5% CO_2. The recombinant viral strain VR-2332 (rVR-2332) and four VR-2332 nsp2 deletion mutants (rΔ727–813, rΔ543–726, rΔ324–523, rΔ324–726) have been described previously (Fig. 1) (Han et al., 2007). The engineered mutants correspond to deletion sizes of 87 amino acids (AA), 184AA, 200AA and 403AA, respectively. Recombinant viruses were sequentially passaged 3–6 times in MARC-145 cells until the individual titers were greater than 1×10^5 $TCID_{50}$/ml. The nucleotide sequences of the amplified recombinant viruses confirmed that no further changes in the nsp2 region had occurred (Han et al., 2007).

2.2. In vivo infection

The in vivo studies were performed at the National Animal Disease Center under its Animal Care and Use Committee approval. Twenty-five healthy pigs at 4 weeks of age were obtained from a herd free of PRRSV and randomly assigned to 6 treatment groups, 4 pigs each to groups 1–5, and 5 pigs to group 6. Groups 1–6 received the following treatments: Group 1 – nsp2Δ727–813, Group 2 – rΔ543–726, Group 3 – rΔ324–523, Group 4 – rΔ324–726, Group 5 – rVR-2332 and Group 6 – sham inoculation (negative control; NC). On day 0, all animals in groups 1–5 were inoculated intramuscularly with the respective virus (1×10^4 $TCID_{50}$/pig) or MEM for NC pigs. Swine were observed daily for general health status. Serum samples were collected from all animals on 0, 2, 4, 7, 9, 11, 14, 21, 28 and 35 days post-inoculation (dpi). On 35 dpi, all animals were weighed and then tracheobronchial lymph nodes were excised and weighed. For each pig, the ratio between the lymph node weight and body weight were calculated (LN/BW ratio). The mean LN/BW ratio for each group was recorded for each group and used as a clinical measurement of viral pathogenesis (Mengeling et al., 2003).

2.3. Serology

Serum samples were examined for serological response with the PRRS 2XR enzyme-linked immunosorbent assay, which utilizes recombinant PRRSV antigens (ELISA; IDEXX Laboratories). A sample was considered positive for antibodies to PRRSV if the sample-to-positive (S/P) ratio was equal to or greater than 0.4. At day 35, convalescent serum neutralization was monitored by immunofluorescence (IFA) (Yoon et al., 1994).

2.4. Serum interferon γ ELISA

Levels of IFN-γ in serum samples at all time points were determined by an ELISA according to manufacturer's recommendations (BioSource, #KSC4022). The assay measures IFN-γ protein

Table 1
Primers used in analyses of viruses. Forward primers indicated by a slash (/) following the name and reverse primers have a slash before the name.

Primer	VR-2332 Nt	Sequence
5′-1050/	1050–1075	5′-TCGCCATGCTAACCAATTTGGCTATC-3′
5′-1264/	1264–1283	5′-GTTGAGCCTAACACGTCGCC-3′
/3′-1711	1711–1736	5′-GACATCCCAGGGGTCACAGTGACAGT-3′
5′-2107/	2107–2125	5′-GACCTGTACCTCCGTGGTG-3′
5′-2167/	2167–2188	5′-CGCCCGCCACGCGTAATCGACA-3′
5′-2392/	2392–2413	5′-CTAACCGCCGTGCTCTCCAAGT-3′
5′-2811/	2811–2832	5′-CCCACCTGAGCCGGCAACACCT-3′
5′-3330/	3330–3355	5′-GCGCGAGGCATGTGATGCGACTAAGC-3′
/3′-3348	3348–3373	5′-GCGTAGCAGGGTCATCAAGCTTAGTC-3′
5′-3666/	3666–3691	5′-CTCCGAGGATAAACCGGTAGATGACC-3′
/3′-3846	3846–3870	5′-CCGGGATCCTTGGTCAAAGAGCCTTTCAGCTTTT-3′
/3′-4257	4257–4287	5′-CCGGGATCCGCCCAGTAACCTGCCAAGAATG-3′
/3′-4300	4617–4635	5′-CTGGGCGACCACAGTCCTA-3′
/3′-5300	5613–5632	5′-GAGTTCTGAAAGCACCCTCA-3′

(0–500 pg/ml) against a standard curve based on recombinant swine interferon gamma. The ELISA plates were read at 450 nm on a SPECTRAmax 190 Microplate Spectrophotometer (Molecular Devices Corporation). Serum levels of IFN-γ were interpolated from a standard curve generated by SOFTmax PRO Version 3.1 software (Molecular Devices Corporation). IFN-γ data were analyzed using a mixed linear model for repeated measures. Linear combinations of the least squares means estimates for serum IFN-γ were used in a priori contrasts after testing for either a significant ($P < 0.05$) effect of PRRSV challenge strain (5 rVR-2332 constructs or sham-inoculated controls), or interaction effect between study day and PRRSV challenge strain. Comparisons were made between groups challenged with each PRRSV isolate and non-challenged controls at each time-point using a 5% level of significance ($P < 0.05$) to assess statistical differences.

2.5. Viremia detection by virus isolation and quantitative Real-Time RT-PCR

To initially determine if the animals were viremic, virus isolation was performed with serum samples collected on all days and lavage fluids at the time of necropsy. Briefly, swine samples (50 μl serum, 200 μl lavage fluid) were inoculated into MARC-145 cells separately and the percent found positive for cytopathic effect (CPE) in each group was calculated (data not shown). Positive samples were then titered on fresh MARC-145 cells. The log value of titer/ml was recorded and graphed for each positive sample. Since all PRRSV constructs or isolates might not replicate in the same manner on MARC-145 cells, Real-Time RT-PCR was performed on identical extracted RNA to further assess PRRSV replication. An in-house standardized quantitative Real-Time RT-PCR, targeting the conserved genomic region corresponding to Ingelvac® PRRS MLV nucleotides 14,947–15,051, was used to detect, in duplicate, the PRRSV RNA copy number in 8 μl of viral RNA purified from serum.

2.6. RT-PCR and nucleotide sequencing

In order to confirm deletion size and ensure accidental contamination with full-length virus did not occur, viral RNA from sera containing virus from 2 animals per group was extracted and subjected to differential analysis using One-Step RT-PCR (Qiagen). In order to detect the virus used to inoculate the animals, the following primer pairs were utilized with 4 μl viral RNA from 2 pigs/group: Group 1 – 5′-2811/3′-3846 (808 bp product); Group 2 – 5′-2392/3′-3846 (936 bp product); Group 3 – 5′-2167/3′-3348 (608 bp product); Group 4 – Primer pair: 5′-2167/3′-4257 (904 bp product); Group 5 – 5′-1050/3′-1711 (674 bp product); NC group – 5′-1050/3′-1711 (no product expected). To detect inadvertent parental virus contamination in Groups 1–4, the following primer pairs were utilized: Group 1 – 5′-3666/3′-4257 (592 bp product); Group 2 – 5′-3330/3′-3846 (517 bp product); Group 3–5′-2811/3′-3348 (538 bp product); Group 4 – Primer pair: 5′-3330/3′-4257 (955 bp product); Group 5 – 5′-2811/3′-3348 (538 bp product); NC group – 5′-2811/3′-3348 (no product expected). Nested PCR was completed on samples from all four pigs from Group 4 at all time points. First round RT-PCR was completed with the primers 5′-2107/3′-4300 (1320 bp product). Second round PCR used 5 μl of the first round product diluted 1:100 and standard conditions using the primer pair 5′-2167/3′-3846 (492 bp product). The primers used for these analyses are listed in Table 1. The individual RT-PCR products were cloned into pGEM-T, amplified in DH5α and then one to two plasmids of each were purified and submitted for sequence analysis to confirm retention of the nucleotide sequence deletion (data not shown). To determine the nucleotide sequence variation after 35 days of infection, the entire nsp2 gene was amplified from two swine in each group. The swine chosen were not necessarily the same swine sampled for deletion verification and preliminary sequence analysis. The PCR products, at 35 day postinfection, were of low abundance and could not be sequenced directly. The products were cloned and amplified, as described above, and then two nsp2 containing plasmids each were submitted for sequence analysis. In this way, a concise indication of clonal variation was obtained.

2.7. Software utilized

Graphpad Prism version 4.0c was used to display HerdChek ELISA and Real-Time RT-PCR results, where the mean and standard error of the mean (SEM) were chosen for representation of individual sample variability within a group. Geneious Pro 3.8.5 (Biomatters Ltd.) was used to examine nucleotide and protein sequences. Alignments were then transferred to Showalign, an EMBOSS (European Molecular Biology Open Software Suite) program embedded in the eBioX 1.5.1 graphical interface (The Linnaeus Centre for Bioinformatics, open source), for suitable display using EBLOSUM90 substitution (Henikoff and Henikoff, 1992).

3. Results

3.1. Immunogenicity and replication of chimeric and parental viruses in pigs

Groups of 4–5 pigs were inoculated with different recombinant viruses (depicted in Fig. 1) or used as a negative control group. Results of anti-PRRSV antibody levels determined by HerdChek* ELISA are illustrated in Fig. 2. All animals were seronegative to PRRSV until 9 days postinfection. By day 14, anti-nucleocapsid antibodies were discerned in Groups 1 (rΔ727–813; 87AA), 2 (rΔ543–726; 184AA) and 5 (rVR-2332), as normally seen with PRRSV field isolates. Group 3 (rΔ324–523; 200AA) swine did not seroconvert until day 21 and one out of four pigs in Group 4 (rΔ324–726; 403AA) seroconverted at day 21 and another at day 35. The two remaining animals in Group 4 did not seroconvert in the course of this study, although one displayed an S/P ratio of 0.387, near the baseline set by the manufacturer for seroconversion (0.400). The data suggested that 87AA or 184AA deletions in the nsp2 hypervariable region 2 carboxyl terminus had no detrimental effect on seroconversion of swine when compared to the parental recombinant clone, but that a 200AA deletion near the N-terminus of the hypervariable region and a 403 AA deletion, which included the majority of the region, retarded the appearance of anti-nucleocapsid antibodies. There were no statistical differences detected in the S/P ratios induced by the recombinant viruses on days 28 and 35, except for the large deletion virus.

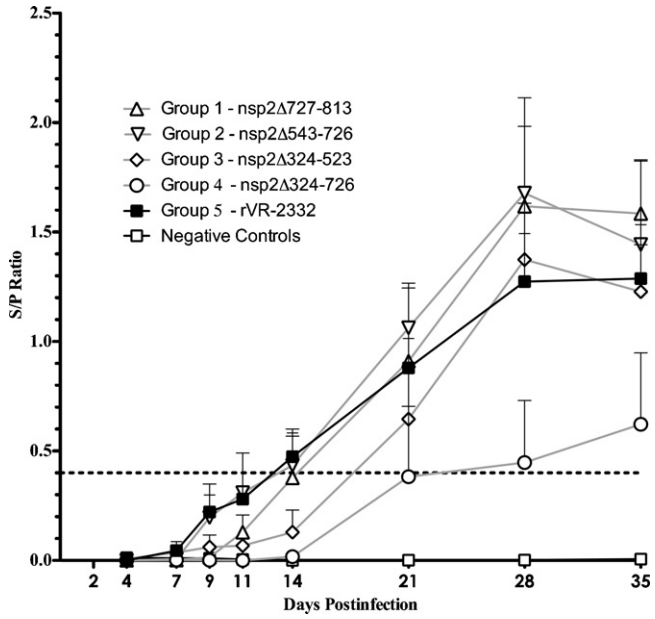

Fig. 2. HerdChek* ELISA, detecting antibodies elicited against PRRSV nucleocapsid protein, were determined for all serum samples and then plotted as the mean and standard error of the mean for each group of pigs at all time points. Serum samples ≥0.4 (indicated by dotted line) were considered positive, as per manufacturer's guidelines (IDEXX).

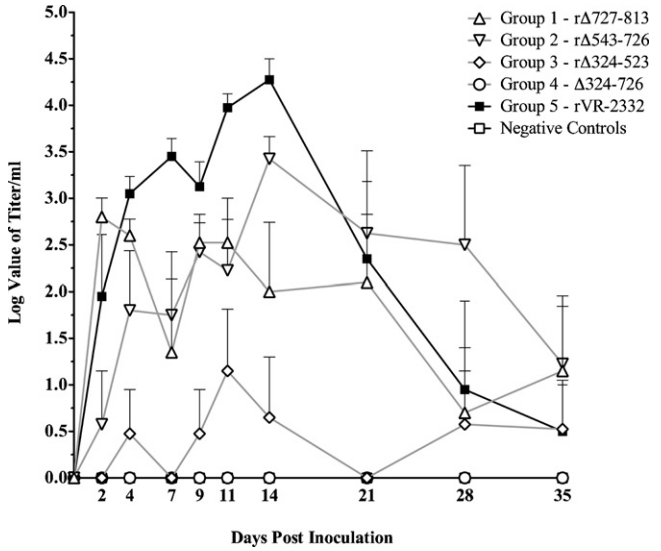

Fig. 3. Virus titer results ($TCID_{50}$/ml) on all virus isolation positive serum samples. Virus isolation was performed with all serum samples and lavage fluids. Positive samples were then titered on fresh MARC-145 cells.

Lastly, no neutralization was detected in serum from any animal (data not shown).

The percentage of serum and lavage samples positive by virus isolation was determined and then titered to assay the ability of the recombinant viruses to replicate in swine (Fig. 3). A delay in viral growth compared to rVR-2332 (native nsp2) was detected for all but Group 1 challenged swine. Group 2 virus growth in swine was only slightly delayed. Virus was isolated only sporadically from swine in Group 3, and never for Group 4 swine. Also evident from this analysis is that although the detectable virus titers from serum had declined by day 35, most infected swine still showed variable amounts of virus in the lung lavage fluid at necropsy, as is typical for PRRSV infection (Table 2).

3.2. Real-Time RT-PCR detection of PRRSV RNA

The fact that some Group 4 swine seroconverted yet did not show evidence of PRRSV replication suggested that virus isolation might not be ideally sensitive. To further evaluate the levels of viral replication, serum samples at all days postinfection for all treatment groups were analyzed by quantitative Real-Time RT-PCR (Fig. 4). Until day 14, there were three discrete levels of viral RNA detected. Swine infected with rVR-2332 had viral RNA amounts typical of PRRSV infection with wt strain VR-2332 (10^4–10^6 RNA copies/ml). Groups 1 (rΔ727–813), 2 (rΔ543–726) and Group 3 (rΔ324–543) swine exhibited viral RNA at 10–100-fold lower

Table 2
Day 35 lung lavage virus isolation and PRRSV Real-Time RT-PCR results.

Group	Virus isolation[a]	Mean	SEM	Copies PRRSV RNA	Mean	SEM
Negative control	0	0	0	0	0	0
	0			0		
	0			0		
	0			0		
	0			0		
1	2.9	3.2	0.6	18,170.0	40,914.1	22,903.6
	1.9			1396.3		
	4.6			105,791.8		
	3.3			38,298.2		
2	0	1.6	1.1	110.4	53,049.7	52,873.5
	1.8			418.4		
	0			0		
	4.5			211,670.1		
3	1.8	1.5	0.5	1388.7	4638.5	2473.1
	0			135.1		
	1.8			11,014.8		
	2.3			6015.5		
4	0	0	0	0	94.2	94.2
	0			376.7		
	0			0		
	0			0		
5	0	1.7	0.7	3386.3	7779.3	5880.9
	1.8			1485.9		
	3.3			25,350.0		
	1.8			895.0		

[a] Log value of titer/ml.

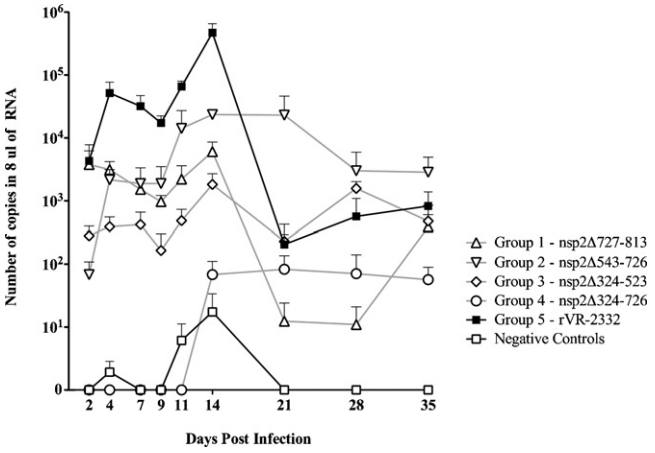

Fig. 4. Real-Time RT-PCR results, performed in duplicate, for all serum samples. Quantitative RT-PCR was used to detect, in duplicate, the PRRSV RNA copy number in 8 μl of viral RNA purified from serum.

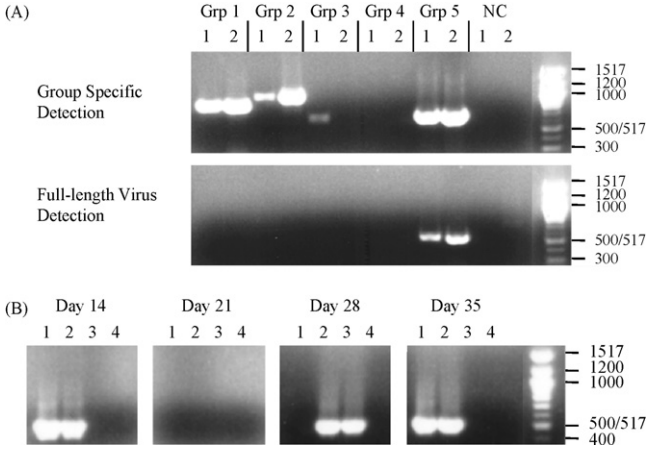

Fig. 5. (A) RT-PCR detection of PRRSV construct used to infect 2 pigs/group (using days 4 and 7 viral RNA from serum samples; day 7 shown) in Group 1 – 808 bp product, Group 2 – 936 bp product, Group 3 – 608 bp product, Group 4 – 904 bp product, Group 5 – 674 bp product, and NC Group – no product expected. RT-PCR to confirm absense of rVR-2332 virus contamination in Group 1 – 592 bp product, Group 2 – 517 bp product, Group 3 – 538 bp product, and Group 4 – 955 bp product. Included were identical RT-PCR conditions for two pigs from Group 5 – 538 bp product (positive control) and NC group – no product expected. (B) Nested PCR was completed on Group 4 at time points that viral RNA was detected by Real-Time RT-PCR (492 bp product).

levels. Lastly, Group 4 (rΔ324–726) showed viral RNA above background only after 14 days of in vivo infection. NC swine showed only background levels of RNA. Also, after day 14, viral detection in all treatment groups was diminished and varied from 10^1 to $10^{4.5}$ copies in 8 μl of purified viral RNA.

3.3. Verification of input virus

At days 4 and 7, viral RNA from 2 pigs/group was tested by RT-PCR with relevant primer pairs to ensure that the virus detected in the different groups corresponded with the input virus and that no aberrant contamination with parental strain rVR-2332 was seen. As shown in Fig. 5A, Groups 1–3 viruses were confirmed to have successfully replicated in swine although at lower levels in Group 3 (barely detectable in pig 2), and there was no evidence of full-length virus growth in any of the deletion virus treatment groups. Nested RT-PCR was used to verify Group 4 mutant virus (Fig. 5B), where the correct size band was variably seen in 3 of the 4 pigs in serum samples from days 14, 28 and 35.

3.4. PRRSV nsp2 protein sequence stability

In order to assess the stability of the nsp2 gene deletion during replication in swine, viral RNA from all pigs/group were extracted from serum taken at day 35. All deletion virus swine sera at this time were first examined for maintenance of nucleotide sequence immediately surrounding the respective engineered deletion site, and the NC swine sera were similarly analyzed using primers encompassing all of the nsp2 deletion regions. All animals retained the correct nsp2 nucleotide sequence (data not shown). After successful RT-PCR amplification of the entire nsp2 gene from 2 pigs/group, 2 DNA products/pig were cloned and 2 of the individual clones were used for nucleic acid sequencing. Silent and mostly single nucleotide changes were scattered throughout the nsp2 gene but it was uncertain as to whether these were due to viral growth in the animal or acquired during amplification and cloning. However, the amounts of nucleotide variation shown in the deletion mutants and rVR-2332 after 35 days of viral growth compared to the starting viruses were similar, suggesting that the mutated nsp2 regions were as stable as the native gene. No universal nucleotide changes from rVR-2332 were seen. Most importantly, all engineered deletions were stable, possessing no additional insertions or deletions in nsp2 (Fig. S1). Some predicted amino acid changes were detected in one or both clones of each mutant after alignment with the inoculating virus sequence. Only four amino acid changes were seen in all sequenced products of the individual mutants [Group 1 – H653Q; Group 3 – G648E and R710W; Groups 2–5 – K1125E (using full-length nsp2 residue numbering)], but all showed 99.2–99.6% amino acid similarity to the respective parent nsp2 sequence and the significance of these changes could not be determined (Fig. S1).

3.5. Serum IFN-γ levels induced by PRRSV infection

In order to establish whether the previous observation that two Type 2 strains induced highly levels of IFN-γ (Wesley et al., 2006) was also seen for strain VR-2332 infection, and to investigate the effect of nsp2 deletions on the IFN-γ levels, all serum samples were assayed. One pig from group 3 (rΔ324–523) had an extraordinarily high IFN-γ level at 7 days (552 pg/ml), which was reproducible. This data point was 4.5 times greater than the next highest serum value detected at any point in the study in any pig (data not shown). When the data value for that pig on that day was removed from the analysis, the group mean ($n = 4$) for that day was 0 (versus 138 pg/ml). The daily standard deviations with this pig excluded from the data set ranged from 0 to 26. The standard deviation for serum IFN-γ levels in all other pigs on day 7 was 5.0, suggesting the value for this one pig was an extreme statistical outlier. Therefore, an analysis of the overall data set was run with the value for this pig on that study day omitted. The effect of removing this data point reduced the treatment group standard error for the least squares mean estimate on day 7 from 21.2 to 7.9. A pattern of IFN-γ response to PRRSV strain VR-2332 emerged when this pig at day 7 was removed from consideration (Fig. 6). The rVR-2332 virus induced a significant biphasic increase in serum IFN-γ levels. The initial increase was observed at day 2 post-inoculation followed by increases peaking on day 11 post-inoculation. These increases were statistically significant on post-inoculation days 2, 11 and 14 and began to decline by day 21. Overall, based on the nsp2 deletion mutant strain of VR-2332 given to pigs, there was a significant effect on elevation of serum IFN-γ levels ($P = 0.0005$). Two groups of pigs receiving different virus constructs (rVR-2332 and rΔ543–726) had significant increases in serum IFN-γ levels above zero over the course of the study. There was a low background level of detectable serum IFN-γ in the non-inoculated control pigs, but rVR-2332 and rΔ543–726 groups were

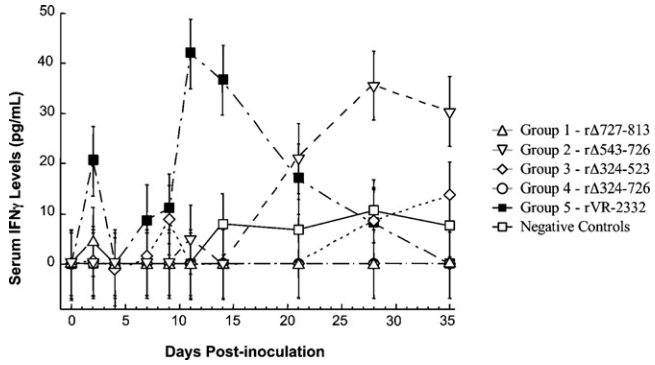

Fig. 6. Interferon γ protein ELISA for all serum samples were analyzed using a mixed linear model for repeated measures.

Table 3
One-way analysis of variants (ANOVA) data obtained from lymph node (LNW) and body weight (BW) comparison on day 35.

Group	*N*	Mean LNW/BW (SD[a])	*P*-value[b]
Negative control	5	0.0738 (0.0207)	
Group 1	3	0.1130 (0.0095)	>0.05
Group 2	4	0.3228 (0.0570)	<0.001
Group 3	4	0.2018 (0.0641)	<0.01
Group 4	4	0.0985 (0.0241)	>0.05
Group 5	4	0.2300 (0.0659)	<0.01

[a] Standard deviation.
[b] In comparison with negative control group.

still different from the sham-inoculated control group ($P = 0.0007$ and 0.04, respectively). Swine in groups 1, 3 and 4 did not produce any IFN-γ above background controls when inoculated with the respective PRRSV deletion mutant.

3.6. Clinical assessment

In order to assess possible changes in virus pathogenicity due to deletion of nsp2 hyvariable region 2 amino acids, all animals were weighed (body weight, BW) at necropsy and then the tracheobronchial lymph nodes were harvested and weighed (lymph node weight, LNW). The resulting LNW/BW fraction, when compared to that of control animals, is a measure of lymph node enlargement (Mengeling et al., 2003). As shown in Table 3, one-way analysis of variance revealed that infection with two deletion viruses, Group 1 and Group 4, did not differ significantly from the negative control animals (>0.05), and showed a significant difference from the rVR-2332 group (<0.05). Since rΔ324–726 (Group 4) did not replicate well in swine, the significance of the lower LNW/BW ratio could not be appropriately evaluated. The other swine groups showed typical lymph node enlargement, a diagnostic indicator of infection with PRRSV. Thus, only one nsp2 deletion mutant of strain VR-2332 did not definitively cause lymph node adenopathy, rΔ727–813.

4. Discussion

Previous work by several investigators had shown that nsp2 deletions in both PRRSV genotypes are naturally seen in the field (Fang et al., 2004, 2007; Gao et al., 2004; Han et al., 2007; Li et al., 2007; Ropp et al., 2004; Zhou et al., 2008). Furthermore, bioinformatic analyses have suggested that nsp2 contains several putative B-cell epitopes (de Lima et al., 2006; Fang et al., 2004), and research has suggested that this protein may be highly immunogenic (Johnson et al., 2007; Mulupuri et al., 2008). Investigators have also shown that insertion of foreign genes such as green fluorescent protein into the nsp2 deletion region has resulted in viable virus, although foreign gene instability has been noted (Fang et al., 2006; Han et al., 2007; Yoo et al., 2004). In our own prior studies, we had determined the essential regions of nsp2 for cell culture passage of PRRSV Type 2 strain VR-2332 (Han et al., 2007). However, no swine disease phenotype has been associated with regions of PRRSV nsp2, although porcine high fever disease of China was originally attributed to a deletion in nsp2 (Li et al., 2007; Zhou et al., 2009, 2008).

In this investigation, we chose four of the previously engineered nsp2 deletion mutants for analysis of viral growth and corresponding disease characteristics in swine. Three deletion mutants possessed in vitro growth phenotypes very close to the parent virus, but the mutant with the largest deletion, rΔ324–726, had been previously found to replicate poorly in cell culture. The targeted section for deletion was located within nsp2 hypervariable region 2 and contained 24 predicted antigenic epitopes (Kolaskar and Tongaonkar, 1990). The recombinant viruses chosen had as few as four (rΔ727–813) and as many as 20 (rΔ324–726) of these epitopes removed. However, other undescribed nsp2 interactions with the immune system, such as the ability to deubiquitinate cell proteins, may have also been compromised as a result of defined nsp2 deletion. We sought to understand the differential effects nsp2 HVR2 deletion had on virus in vivo growth kinetics and potential correlations with the antibody responses, number and position of putative B-cell epitopes, the innate immune response protein INF-γ and particular clinical disease manifestations over the 35 day experiment.

We found that all of the mutant viruses replicated in vivo, and all but the virus with the large deletion (rΔ324–726) induced a strong IDEXX anti-nucleocapsid antibody response. The response mirrors the viral growth in swine as measured by virus isolation, with rVR-2332 reaching the highest titers and gradually decaying over time. The three smaller deletion mutants (87, 184 or 200 AA removed) grew approximately 10–100-fold less vigorously, but were nonetheless able to induce antibodies to the nucleocapsid protein rapidly. A notable delay in antibody response was seen in three of four animals infected with the mutant harboring the largest nsp2 deletion, but was the first indicator that this virus had indeed replicated in swine. We also examined PRRSV RNA copies detected by Real-Time RT-PCR, and noted that this measurement approximated the virus isolation results, but was more sensitive to viral nucleic acid and was able to discern a small and stable amount of the large deletion mutant viral RNA at later times in infection. The shape of most curves in both assays suggests a cyclical nature for PRRSV growth. When the lung lavage fluids were examined by the same assays, it appeared that the viruses infecting Group 1 (rΔ727–813, 87 AA deletion) and 2 (rΔ543–726, 184 AA deletion) animals were able to persist in the lungs as well as native VR-2332 nsp2. We also sought to determine if the viral mutants were genetically stable during replication in swine, and found that although there were periodic silent nucleotide changes and some amino acid changes, no further editing of the engineered deletions in nsp2 was detected, suggesting the virus adapts readily to mutation in this hypervariable part of the replicase protein, as has been amply noted in the field (Fang et al., 2004, 2007; Gao et al., 2004; Greiner et al., 2000; Han et al., 2007; Li et al., 2007; Ropp et al., 2004; Zhou et al., 2008). Sequence analysis of the entire genomes of these interesting viral mutants after growth in swine, which are underway, will have to be completed in order to examine changes that may have occurred outside of the nsp2 region.

IFN-γ, an important cytokine produced during the early stages of infection by macrophages, natural killer (NK) cells, T cells and alternative cells types is a mediator for cellular immunity. Previous work reported that PRRSV Type 2 strains JA-142 and SDSU-73 induced release of IFN-γ into the serum of infected animals whereas two other viruses, porcine coronavirus and swine influenza virus, did

not induce comparable IFN-γ serum levels (Wesley et al., 2006). In order to examine whether this effect was seen after infection with PRRSV strain VR-2332 and to test if deletion of parts of the nsp2 HVR 2 would alter the response, the serum samples from the present study were assayed in the same manner, but with additional time points included. Again, we noted the paradoxically high levels of IFN-γ in serum of swine that had been infected with full-length recombinant virus rVR-2332, but it also appeared to be biphasic in nature. However, only one deletion mutant, rΔ543–726, was able to generate a similar response in swine, although it was delayed by approximately 2 weeks. The other mutants showed no discernable rise serum IFN-γ above the control group. When compared to the virus load diagrams, the peak of IFN-γ appeared to precede the peak of viral titer or RNA load detected, which may have revealed a unique feature of infection caused by this persistent virus. The role of this interesting cytokine in the serum of PRRSV infected animals is thought to be critical but is still poorly understood. In addition, the effect of virus growth kinetics and the amount of circulating virus on inducing serum INF-γ must be further studied.

Although no obvious clinical signs were noted in this study, we sought to examine whether deletion of separate polypeptide sequences of nsp2 would have an effect on any disease parameter. LNW/BW ratios revealed that only one mutant, rΔ727–813, caused a discernable decrease in lymph node enlargement over that seen with rVR-2332. Thus, the smallest deletion with only 87AA acids removed from nsp2 was able to induce diminished clinical disease whereas the larger upstream 187AA and 200AA deletions had no moderating effect. Bioinformatic analysis of the 87AA region did not yield any noticeable protein motifs, although the high proline content (10%) suggests a highly structured protein region. Finally, when comparing the putative antigenic regions discussed above, the regions containing 10 such motifs did not reduce the lymphadenopathy, while a region with only 4 domains significantly decreased this measurable sign of pathogenicity. Whether the reduction of the predicted B-cell epitopes results in an attenuation/exacerbation of other clinical signs must be examined using alternative PRRSV strains. However, the viral mutants described in this report will be extremely useful in the search for other modulators of PRRSV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.07.024.

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